Engineering 3D Collagen Tunable Platforms for Mechanobiology Studies

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Master of Science

By

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ABSTRACT

Construction of scaffolds is crucial for tissue engineering applications. Three dimensional (3D) scaffolds provide extracellular matrix analogs that function as suitable platforms for cell infiltration and physical supports to guide their differentiation and proliferation into the targeted functional tissue or organ. An ideal scaffold used for tissue engineering should possess excellent biocompatibility, microstructure and porosity adapted to cell invasion, controllable biodegradability, and suitable mechanical properties. Collagen is among the most promising biological materials for scaffold fabrication. It has numerous applications in tissue engineering such as nerve, bone, cartilage, tendon, ligament, blood vessel, and skin repair. In this study, we report the design, synthesis, and characterization of porous and bioactive type I collagen scaffolds via an ice-templating method, and we detail how mechanics, morphology, and protein contents of the scaffolds were tuned to make them suitable for mechanobiology studies.

Scaffolds were generated by varying collagen concentration, freezing temperature, and mold material/shape. Their morphological and mechanical properties were assessed via Scanning Electron Microscopy, Hg Porosimetry, and Dynamic Mechanical Thermal Analysis. Our data indicate that the best control over scaffolds properties was achieved when using the Teflon rectangular molds and freezing temperatures of -10°C while varying collagen concentration: Average pore size decreased from 214 μm to 35 μm and compressive modulus increased from 154 Pa to 1720 Pa when collagen concentration was increased from 0.5 wt.% to 1.25 wt.%. This trend tended to less pronounced when lower freezing temperatures were tested. Additionally, the scaffolds were coated with fibronectin (a protein from the extracellular matrix that promotes cell adhesion) to investigate the

additional effect of molecular conformation on cell behavior. Collectively these data suggest that cell adhesion and proliferation can be coarsely controlled by scaffold morphology and mechanics and then finely tuned by protein conformation.

Our research lays the groundwork for future investigation of the different methods to fabricate tunable collagen scaffolds and control the synergistic effects of collagen with other proteins present in the cellular/tissue micro-environment, which are crucial to govern cell-matrix interactions in mechanobiology studies.

BIOGRAPHICAL SKETCH

Yifan completed his undergraduate studies in the Macromolecular Materials and Engineering department at Sichuan University in June 2015. He then came to Cornell to pursue an M.S. as he continued to work in biomaterials. He began researching the fabrication of collagen scaffolds for would healing purposes in Dr. Delphine Gourdon's laboratory in the fall of 2015 for his Master degree.

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I would also like to thank my parents, Pengpeng Hou and Hongwei Li, who worked tirelessly over the years to make sure I was loved, cared for, and educated. Without your help, I never would have made it this far.

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1. Introduction

1.1 Introduction to Porous Materials

Porous materials are applied in many applications such as catalysis, gas storage/separation, filtration and biomedical treatment due to their low density, relatively high surface area to volume ratio and appropriate mechanical properties **[1]**.

Besides the surface energy resulting from the formation of pores within the structure, there is also the research of unoccupied space (in the absence of an intruding liquid) that is filled with air. This porosity introduces a means through which another material (whether solid, liquid, or gas) may be introduced into the bulk structure. Additionally, the formation of open pores reduces the density of the overall structure, thereby aiding the use where the structure's lightweight nature and tunable mechanical properties offer improvements in performance, cost, and efficiency.

Along with several applications which porous materials are of great benefit, such as gas storage, filtration and separation, energy storage and conversion, the application of porous materials in biological applications has drawn great attention during recent years **[2]**. Using the porous 3D scaffolds for cell culture provides an environment that nurtures biologically relevant behavior in vitro. The tunability of pore structure and mechanical properties of these scaffolds allow us to achieve fine control of cell behavior that is useful to biomaterials and mechanobiology research.

Pore size, as defined by the International Union of Pure and Applied Chemistry (IUPAC), is categorized as follows:

- Macropores: > 50 nm diameter

- Mesopores: 2-50 nm diameter

- Micropores: < 2 nm diameter

Porous materials may contain pores of various size and shape. Contributions to surface area and pore volume are highly dependent on the pore diameter and morphology. Smaller pores offer high surface area but low total pore volume, while larger pores offer higher volumes but lower surface area **[3]**. The pores arrangement in 3D space may possess specific order or randomly distributed as anisotropic arrangement. Based on the application for which the porous structure is to be used for, it may be desirable to direct and tune porosity in the material for better performance.

Porous materials can be fabricated in various ways that allow structural control with differing extents of relative feasibility. Polymerization of a monomeric compound can get interconnected porous structure and subsequent crosslinking can help to improve the mechanical properties and overall robustness. This method is a widely used to make structures with high mesoporous surface area and interconnected porosity, however, the extent to which the pore sizes and isotropic degree may be tuned is restricted. Ordered porous materials can be prepared from the use of block copolymers; this method is a powerful method to create unique, complex morphologies by utilizing microphase separation that results from the self-assembly of polymer domains. The use of sacrificial templates within the block copolymer allows for the formation of an ordered, interconnected structure upon processing of the sample **[4]**. Although the pore morphology can be finely tuned by changing the volume fractions of individual components, this method can be expensive and difficult to scale up for industrial applications.

Other methods such as high internal phase emulsion polymerization can also be used to produce structures with interconnected pore structure. However, the defect of this method is that materials are sometimes mechanically weak structures with relatively low porosity. Porous structures may also be fabricated through the use of soft or hard templates that act as structure directing agents. Soft templates introduce porous structure either by forcing the aggregation of solid contents into a well-defined structure, or by directing the formation of structure through the development of crosslinking so that, when the templates are removed or disassembled, a porous structure still remains intact structure. Hard templates shape the material into the desired structure and are removed to produce a negative replica of the template itself. Hard templating is an effective method of preparing porous structures, however the template removal adds extra time and expense for the materials synthesis process **[5]**.

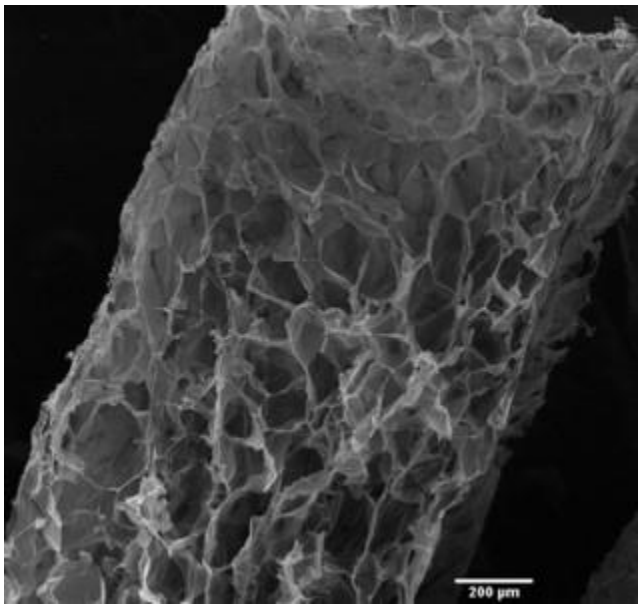

Figure 1.1.1: Freeze-cast, macroporous structure prepared from collagen colloidal solution. Scale bar: 200 µm

Freeze-casting is a method of hard templating in which interconnected, macroporous 3D structures (**shown in Figure 1.1.1**) are prepared from liquid dispersions with relative ease and cost. The first step is the freezing process from liquid to solid, the cold temperature crystallizes the water, introducing an ice front that moves along the suspension. As the crystals are formed, they expel the colloidal particles from the solidifying water. Subsequently, the colloidal particles are concentrated within these inter crystals regions.

And the second step is the freeze-drying process, after the freezing process, the two distinct phases are structured, whereby the ice is the sacrificial template. So subsequently, it is simply a matter of using high vacuum to sublimate of ice without affecting the structured phase. Sublimation could avoid the structure collapse caused by the capillary force. After some additional post processing, such as the chemical crosslinking or de-hydrothermal treatment to increase the mechanical properties of the scaffold structure, interconnected porous scaffolds can be easily fabricated through freeze-casting method.

In 2009, Sylvain Deville et al. further investigated crystal growth and particle movement in simple colloidal dispersions systems frozen at a fixed rate using fast X-ray computed tomography **[2]**. Based on this method, Deville could probe the development of structure change in solidified dispersions through simple image analysis, as the particle phase and ice phase interact differently with the X-rays used for the sample imaging. Lamellar structure could be generated on a cold surface with freeze-casting; representative images of the samples are presented in **Figure 1.1.2**

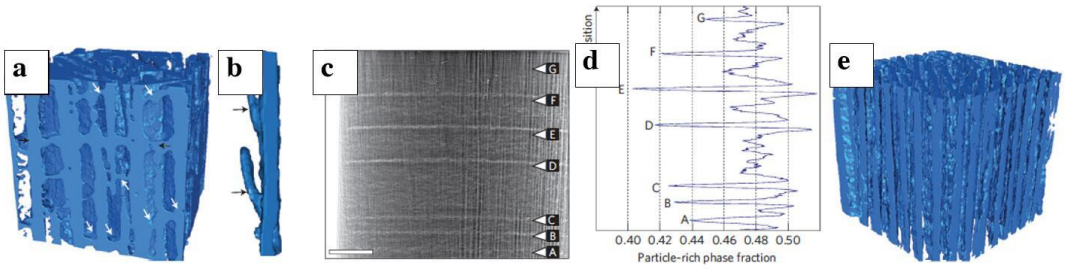

Figure 1.1.2: Probe of the development of instabilities in frozen colloidal dispersions. A: 3D reconstruction (360 µm x 360 µm x 360 µm) of a solidified solution containing 0.2 µm sized particles. White arrows indicate the location of localized instabilities formed in the structure. B: Side view of instabilities formed during freeze-casting. C: Radiograph of a sample showing the presence of instabilities, viewed as light horizontal streaks in the image. D: Plot of particle fraction present at the location of each instability. E: 3D reconstruction (360 µm x 360 µm x 360 µm) of a solidified dispersion containing 1.3 µm sized particles **[2]**

Development of instabilities on lamellar crystals led to the formation of cell-like microstructure originated from the nucleation of homogeneous ice crystal in constitutionally supercooled liquid ahead of the ice front. Nuclei that are constructively oriented will undergo rapid growth within that region, leading to the formation of large crystals that are oriented perpendicular to the temperature gradient. Subsequently, the morphology transitions took place, more uniform cellular structure would replace the anisotropic lamellar microstructure **[6]**.

Growth of a homogeneous lamellar structure is also dependent on the particle size and the speed at which the solidification took place. Therefore, a phase diagram (**Figure 1.1.3**) over the experimental space may be created-at high front velocities and large particle sizes, entrapment of the particles in the ice phase occurs. Adequately low front velocities lead to complete rejection of the particle phase. Only at intermediate front speeds, the formation of interpenetrating networks of ice and particles may occur.

The locations of metastable and unstable morphologies are indicated with green and red regions on the phase diagram respectively **[2][6]**.

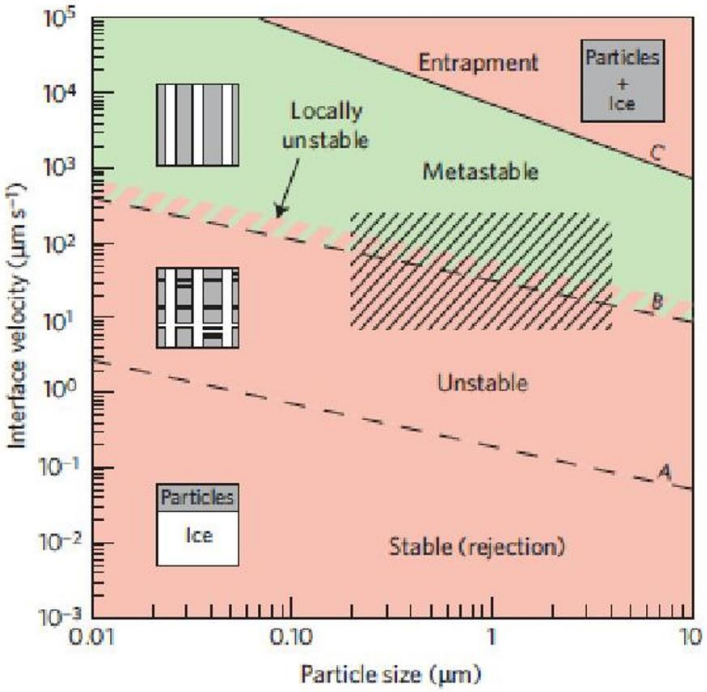

Figure 1.1.3: Stability diagram of morphologies achieved upon solidification of a colloidal dispersion at a range of interface velocities and particle sizes. The hatched region in the diagram specifies the experimental conditions explored in Deville's research

This work will be mainly concerned with the synthesis of porous structures via freeze-casting method, as well as tuning the structure and properties in the context of biomaterials and mechanobiology studies.

1.2 Freeze-Casting for Generation of Porous Materials

The phase separation with solidification of a colloidal dispersion can be explored for the formation of porous structure comprised exclusively of the dispersed phase. Freeze-drying of the solid structure leads to the sublimation of the ice phase, thereby preventing structural

collapse because of strong capillary forces that developed during melting process. Consequently, the remaining structure has pores that are negative replicas of the ice phase created during the freezing process. This method has been used to prepare porous structures from a variety of precursors such as ceramics, biomolecules, polymers, and composite materials **[7]**. Freeze-casting produces macropores of which the size and morphology are a direct result of the freezing conditions and dispersion composition used. In 2007, Deville et al. presented a study on the mechanical properties and microstructure of freeze-casting hydroxyapatite/alumina composite materials, showing that for an equivalent inorganic/organic solid content, freeze-cast structures presented superior mechanical resistance as compared to the natural materials such as nacre. The microstructure, especially the thickness of the pore walls was significantly influenced by the speed of the ice fronts. Faster growth of the ice phase gets thinner pore walls. Furthermore, the thickness of pore walls was influenced by the liquid content of the slurry to be freeze-cast, samples with lower liquid content had thicker pore walls in the final structure. The mechanical properties of these structures are controlled by microstructure and the infiltration of structure with additional materials and post treatment after freeze-casting **[8]**. Because of their excellent biocompatibility and mechanical properties, these freeze-casting structures are promising candidate materials for biomaterials research and applications.

1.3 Structure Property Correlations in Freeze-Casting Materials

Despite numerous previous research efforts, no exact design requirements exist as to which the combination of overall porosity, average pore size, mechanical properties, pore connectivity, material surface roughness, surface chemistry in a freeze-cast scaffold is best suited for each respective tissue or organ **[9]**. The results of ongoing systematic studies of

such structure property correlations are expected to provide future direction for tissue engineering development.

However, the first structure-property linkages relating material structure to mechanical performance for freeze-casting materials have been established. These correlations can already be influenced by slurry preparation as the slurry composition and formulation have a significant effect on the scaffold's microstructure and mechanical properties. In polar solvents, such as water, surface charges on particles are favorable because they support particle dispersion and thereby improve the overall homogeneity of the entire sample **[10]**. Indirectly, they also affect other important processing parameters, such as the viscosity of the slurry and the particle sedimentation, as these depend on the particle size. Based on that, it is conceivable to adjust surface charges through, variations of the liquid's pH and surface oxidation state **[11]**.

Additives in the slurry could be utilized to control the surface structure and roughness of the lamellae created during the freezing process. The shape of the pores created by the liquid carrier depends on the crystal type and growth characteristics with which it solidifies.

The amount of solid content in the slurry determines the overall porosity of the scaffold sample; the higher the loading is, the lower the overall porosity achieved. The honeycomb-like structure created by directional solidification results in the mechanical properties that are significantly higher than those achieved by non-directional freezing and lyophilization or other processing methods that result in a foam-like material. Both strength of honeycombs loaded and the compressive modulus parallel to the long pore axis are predicted to scale linearly with the relative density of the material. For a given overall porosity, the lamellar thickness and spacing in a freeze-cast material can be controlled. An

increase in the cooling rate to achieve an increase in the freezing-front velocity results in smaller lamellar spacing. As a common tendency, a decrease in the lamellar spacing results in an increase in compressive strength **[12]**. However, different trends have also been observed, mainly in the freeze-cast polymers.

Since the porosity generated by freeze-casting is highly interconnected, it is possible to create hierarchical microstructures by sequential freeze casting treatment, thereby exploring the potential to introduce another level and another direction of porosity into the material. The high connectivity also makes it possible to coat the sample once or several times with proteins and to infiltrate it with another material. This combination offers great potential to integrate into the biomaterial growth factors and other biochemical cues needed to promote tissue and cell ingrowth **[13]**.

1.4 Controlling the Structure of Freeze-Casting Materials

Based on the previous research, the porous scaffold fabricated by freeze-casting method could be controlled by changing the processing parameters such as **[14]**:

Solvent used: Solvents besides water can be used for freeze-casting liquid carriers, only if they can be removed from the structure through sublimation with minimal impact on the pore structure produced via freezing process. By varying the solid content of the dispersion, structures with controllable porosity (pore size and wall thickness) can be obtained. Solvents such as cyclohexane, tert-butanol, and glycerol are also in use for the synthesis of freeze-cast structures.

Solvent viscosity: The viscosity of the freezing-casting solution has been shown to impact the mechanical properties of the resulting freeze-cast monolith. Increased viscosity of

aqueous dispersions comprised has led to improved compressive modulus of the macroporous structure after drying and post treatment.

pH of dispersant: Previous research shows microstructure observed in freeze-casting monoliths prepared from materials such as TiO2 and graphene is greatly influenced by the pH of the colloidal dispersion prior to solidification.

Temperature: As previously mentioned, the temperature used to freeze a dispersion of particles can influence the shape of ice crystals by causing different extents of constitutional supercooling. Therefore, it is possible to create pores with a variety of morphologies, from lamellar structures to cellular structures.

Dispersion concentration: By increasing the amount of solid content in the dispersion, the pore wall thickness may be tuned deliberately to improve the mechanical properties (through higher solid content loading) or porosity.

As mentioned above, it is possible to achieve the fine control over the mechanical properties and microstructure of the porous monolith prepared via freeze-casting through the cautious tuning of processing parameters.

1.5 Freeze-casting for the Facile Synthesis of 3D Porous Collagen Scaffolds

In this work, freeze-casting will be presented as a means of creating porous scaffolds from a variety of type I collagens for biomaterials and mechanobiology applications. In Chapter 2, we will address the synthesis and properties of freeze-cast Bovine type I collagen scaffold, later used as scaffolds for cell growth. Chapter 3 will focus on the results' presentation and the examination of the influence of (i) freeze-casting method, (ii) solvent concentration, and (iii) freezing temperature on the pore structure and mechanical

properties observed in the scaffolds. Finally, in Chapter 4 and 5, conclusions, challenges and opportunities for the further implementation of freeze-casting in the synthesis of materials for academic research and industry applications will be addressed.

2. Porous Type-I Collagen Scaffold for Mechanobiology Studies

2.1 Materials for Mechanobiology Studies

The close intersection of materials science and biology study has led to the focused research of materials that are engineered specifically for mechanobiology studies. In the past, flat two dimensions platforms were used for the studies of cell behavior; however, since they do not accurately reflect the typical living environment of cells found in vivo, a more complexed and advanced platform has to be established. Cell adhesion to a surface requires the generation of tension within its cytoskeleton around the extracellular matrix, and consequently the cell is able to respond to the mechanical cues and signals of its surroundings. Typically, two-dimensional (2D) environment does not offer the same surroundings to the cells - this would lead to different cell behavior observed in vitro-when it compared to more physiological related and complex three-dimensional (3D) environments. As an example, it has been shown that the generation and development of focal adhesions (structures that mediate the attachment of cells to a substrate) greatly differs between 2D and 3D environments. The development of 3D matrices to be used for these types of studies becomes the urgent need for the cell behavior observations (e.g. differentiation, migration, protein expression) **[15]**.

What are the most eminent features of a three-dimension platform to be used for the research of mechanobiology? Firstly, the mechanical properties of the scaffold should be representative to those observed in vivo environment. This means that the stiffness such as compressive modulus of the matrix is important - previous study shows that neural cells require a much more compliant matrix surface (in vivo: 260-490 Pa), as compared to the one used for the growth of bone cells (in vivo: 2-20 GPa) - with regard to the observation

of biologically relevant behaviors **[16]**.

Additionally, the scaffolds must possess an interconnected porous structure so that they can appropriately size to accommodate cell initial infiltration, subsequent invasion, proliferation and differentiation. Importantly, the scaffolds should be biocompatible so that, when their material breakdowns and degrades, it should not release byproducts which are cytotoxic or that will influence the behavior of the hosted cells in unpredictable or undesirable patterns. Still, there are additional considerations concerned with the feasibility/usefulness of tissue engineered 3D structures (e.g. cost of scaffold production, irritability for local and surrounding tissues, ease with which the production of scaffold may be scaled up); nevertheless, all these factors are secondary in this research.

Nowadays there exist a large number of approaches toward the production of scaffolds that are appropriate for the studies of cell behaviors **[17]**. In some cases, native extracellular matrix (ECM) is derived from existing tissue (via detergent-mediated decellularization) and is then incorporated with new cells; however, the expense and effort coming along with this approach makes it highly unpractical for large-scale applications. The research and development of inexpensive, synthetic scaffolds that may be used to mimic and replace ECM, therefore, becomes the great interest to the scientific community. Scaffolds made from hydrogels (derived from collagen, polyacrylamide, etc.) offer tunable porosity and appropriate mechanical properties through modified polymerization conditions, cross linker application and additive incorporation. Also, 3D scaffolds with controllable structures may be prepared with sacrificial templates. Polymer-based structures may be prepared via electrospinning; however, this approach does not offer fine control of porosity for the final structures **[15]**.

Finally, methods such as solvent casting/leaching, extrusion, thermally induced phase separation, and 3D printing have also been explored as means of creating interconnected porous structures **[18]**. Great interest from scientific community has been put in the research of structures made from natural polymers and proteins to mimic and direct cell behavior.

Collagen, in particular, type I collagen, with a wide range of useful features such as low antigenicity, excellent biocompatibility and appropriate mechanical characteristics, has been frequently used as the main material for scaffold fabrication **[13]**. Collagen scaffolds may be formed by freeze-casting of their aqueous solutions, usually an organic acid is added to encourage the formation of a dendritic ice crystal network without side-branching. Following the ice phase sublimation, a porous structure with exactly the same morphology as the ice crystals is created within the monolith. This makes freeze-casting a feasible way to control the properties of the scaffold topography through the processing parameters adjustment **[19]**. Developing a 3D collagen scaffold platform for the applications in mechanobiology would highly boost the research within this field.

In this study, synthesis and characterization of porous and bioactive collagen scaffolds from type I fibrillar collagen (via freeze-casting method) and their application in mechanobiology research are reported. The type I collagen scaffolds developed offer tunable pore size, mechanical properties, and show enhanced bioactivity when combined with fibronectin, another ECM major component **[20]**. As we know, the mechanical and structural properties of the collagen scaffold can direct cellular activity within a tissue-engineered construct. The pore sizes and mechanical properties of collagen scaffold is vital to the cell initial adhesion and further migration into the internal area of scaffold complex;

therefore, the ability to manually control the various properties of scaffold is a very useful tool for future mechanobiology research **[18]**. Extension of this work to 3D collagen-fibronectin scaffold systems would provide a means of controlling protein conformation and cell deposition in an environment that better resembles in vivo settings. Freeze-casting is a facile means of creating porous structures with tunable morphology and mechanical properties; consequently, the work presented in this chapter is concerned with the use of type I collagen dispersions for the preparation of 3D collagen scaffolds, with porosity well-suited for cell development and growth.

2.2 Collagen as a Structural Material

Type I collagen is the most abundant collagen of the human body including tendon, ligament, dermis, and blood vessel, and is the primary determinant of tensile properties, it can also form large, eosinophilic fibers known as collagen fibers. In this study, three-dimensional (3-D) matrices were prepared from type I collagen, the predominant compositional and structural component of connective tissue in ECMs **[11]**. Type I collagen is present in scar tissue, the end product when tissue heals by repair, as well as tendons, ligaments, the endomysium of myofibrils, the organic part of bone, the dermis, the dentin and organ capsules.

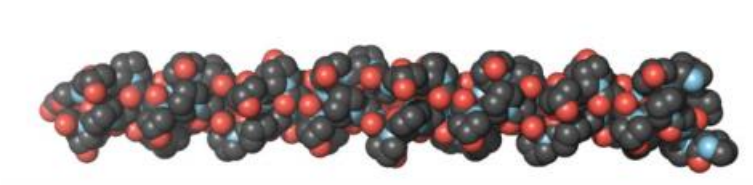

Figure 2.2.1: High-resolution crystal structure of a collagen triple helix

Type I collagen biological scaffold has been utilized in a variety of tissue engineering

applications including skin, peripheral nerve, muscle and cartilage **[21]**. These scaffolds are extremely biocompatible with non-toxic degradation products, have a high porosity of 99.5% with an interconnected pore structures allowing for the infiltration of nutrients and the subsequent removal of waste products. These characteristics make the type I collagen scaffold extremely attractive for use in wound healing and the research of mechanobiology **[22]**. An additional advantage of these scaffolds is that their microstructural architecture, such as the pore size, can be controlled by varying several parameters during the fabrication process. These scaffolds are fabricated through a lyophilization or freeze-casting process whereby a suspension of collagen in acetic acid is cooled to a final temperature of freezing **[23]**.

These collagen scaffolds were then applied to in vitro studies for cell behaviors (in particular cell invasion/migration, proliferation and adhesion) and it was found that the pore size significantly affected cell attachment within the scaffolds with the smallest mean pore size leading to the highest levels of attachment. It was hypothesized that this was due to an increased surface area producing a higher ligand density for cells to attach. Pore size is thus an important parameter of tissue engineered scaffolds as it defines the specific surface area and ligand density that is available for cells to bind and migrate on. It has been proposed that pore sizes need to be large enough for cells to migrate into the middle part of the scaffold, but small enough to keep a high specific surface area and many investigations have found that cell attachment and the corresponding cellular behavior is significantly affected by pore size **[24]**.

An important goal in the research of cell behavior in vitro environment is the fabrication of a biologically relevant matrix in which the cells to be studied will invade and proliferate.

A 3D platform made by the freeze-casting process allows for the adhesion of seeded while providing sufficient space for growth, migration, and differentiation **[25]**. Because the freeze-casting method provides the tunability of scaffold porosity and mechanical properties, it is possible to develop a scaffold with optimized mechanical properties and morphology for a specific given application **[26]**.

In this study, we used of a modified freeze-casting technique to prepare macroporous structures for mechanobiology studies. To achieve sufficiently large pore sizes, we utilized lower concentrations of collagen solutions, combined with higher freezing temperatures.

2.3 Control of Freeze-Casting Collagen Structure

Three parameters were expected to be crucial in controlling scaffold microstructure **[27]**:

- **Precursor composition:** The collagen concentration of the suspension can be easily varied by controlled addition of the solvent. We anticipate that increasing the concentration of collagen content will lead to smaller pores because of either higher pore density or increased cell wall thickness.
- **Freezing rate and nucleation temperature:** The temperature at which the sample starts to freeze greatly impacts the rate of nucleation and ice growth. To investigate this phenomenon, we have examined the microstructure resulting from different freezing temperature. We anticipate that rapid freezing will lead to a greater number of ice nucleation sites in the solvent and consequently a higher density of pores in the material. Conversely, we anticipate that slower freezing rate will yield larger ice crystals, which will result in a monolith with larger pores. Also, lower freezing temperature would lead to smaller pore sizes because of the limitations of ice crystal

growth.

- **Mold composition:** Because the thermal conductivity of the mold could affect how rapidly the suspension inside can be cooled, its composition can also influence the solvent freezing behavior, thereby changing the subsequent microstructure of the collagen scaffold. Molds made from materials having high thermal conductivity lead to rapid freezing of the material at the mold-suspension interface, while molds made from low thermal conductivity materials allow the suspension to freeze at roughly the same rate throughout. Previous studies in our group examined the difference between freezing behavior in brass molds (with high thermal conductivity) and polytetrafluoroethylene (PTFE), which has much lower thermal conductivity. We therefore anticipate that samples frozen in highly thermally conductive molds would have a sharply different microstructure when compared to the samples frozen in thermally insulating molds **[28]**. Thus, modification of these parameters (individually or in concert) should allow for the facile fabrication of collagen scaffold with various tunable porosity and mechanical properties.

2.4 Hydrophilicity of Collagen Scaffolds

The reactivity of collagen hydroxyl groups provides a way in which a monolith can readily incorporate water (via hydrogen bonding) into its bulk structure, possibly leading to lower porosity as the result of scaffold swelling **[29]**.

Additionally, wetting may also influence the mechanical properties of the scaffold. The dry sample may be considered a two-phase material, with an interconnected network of collagen throughout the sample and air in the void area. In this case, it is possible to

establish a relationship between the elastic modulus of the monolith and its porosity, where the nonporous material would have the highest compressive modulus and the modulus would decrease continuously with the increasing porosity. However, this is not necessarily the case for hydrated collagen scaffolds, where water invasion would result in higher mobility of the collagen chains. Prior studies have shown that the modulus of wet hydrophilic samples is significantly lower than that of a dry sample, due to the greater mobility of the collagen chains in response to the applied stress **[30]**. We would therefore propose that fully hydrated samples would be significantly more compliant than their dry counterparts. Consequently, control of the structure and mechanical properties is highly important for the collagen scaffold fabrication **[24]**.

2.5 Chemical Crosslinking of Scaffold Samples

In order to increase the mechanical properties and stability of collagen scaffold, appropriate crosslinking method should be utilized into the scaffold system. Chemical crosslinking within the type I collagen is widely used for the scaffold preparation. From the proline, hydroxyproline and glycine shown in the **Figure 2.5.1**, there are many carboxylic acids and primary amines in the triple helix collagen structure, subsequently, finding a useful chemical reagent to induce the amide bond formation will increase the stability and mechanical properties of the final collagen scaffolds. Here, a carbodiimide compounds named EDC, 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide hydrochloride was used as the main crosslinker and a N-hydroxysuccinimide abbreviation (NHS) was used to improve its efficiency **[31]**.

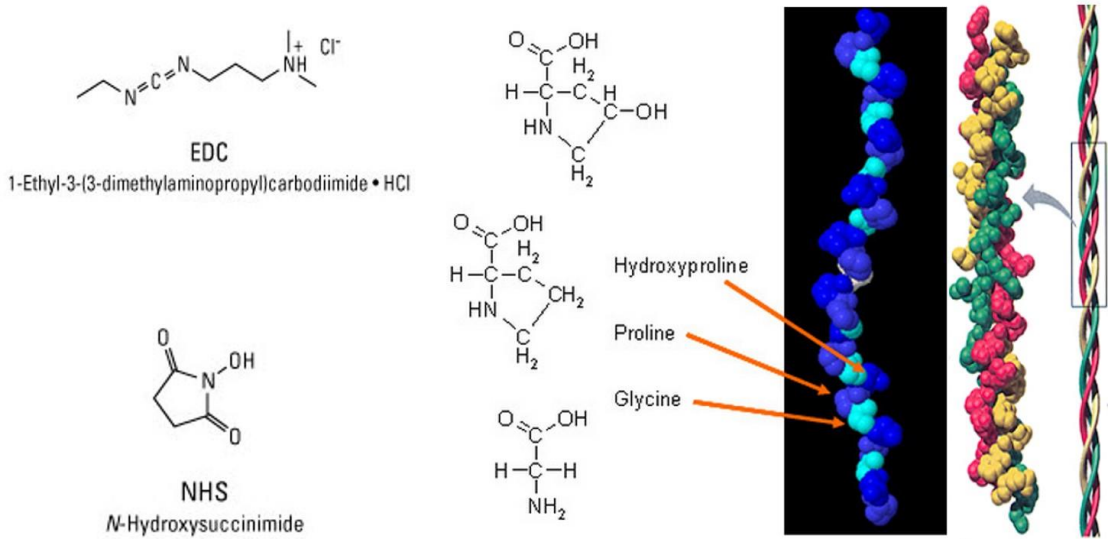

Figure 2.5.1: Crosslinking region of collagen structure

Here the carbodiimide EDC crosslinking reaction scheme is shown in **Figure 2.5.2**. EDC reacts with carboxylic acid groups to form an active intermediate, which is easily displaced by the nucleophilic attack from primary amino groups in the reaction mixture. The primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative. Because no portion of their chemical structure becomes part of the final bond between conjugated molecules, carbodiimides are considered zero-length carboxyl-to-amine crosslinkers. So, the crosslinking process will not compromise the biocompatibility of the final collagen scaffold. N-hydroxysuccinimide (NHS) or its analog (Sulfo-NHS) is often included in EDC coupling protocols to improve the efficiency by create dry-stable intermediates. EDC couples NHS to carboxyls, forming an NHS ester that is considerably more stable than the original intermediate while allowing for both sufficient and efficient conjugation to primary amines at physiologic pH condition **[31]**.

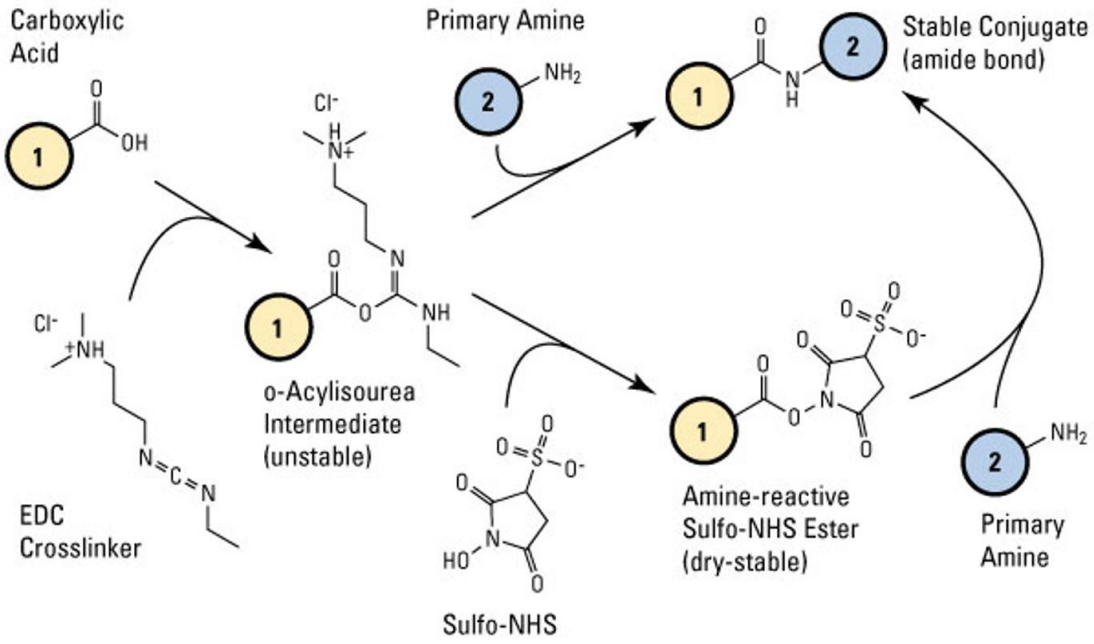

Figure 2.5.2: Reaction Chemistries of EDC/NHS Crosslinker

2.6 Scaffold Synthesis and Characterization

2.6.1 Preparation of Type I Collagen Scaffold via Freeze-Casting Method

Collagen monoliths were freeze-cast in polytetrafluoroethylene (PTFE) molds with either rectangular or circular shapes/wells, which were cleaned and dried prior to use. Two types of freezing method were used for these experiments: (i) controlled freezing rate and (ii) controlled nucleation temperature, in order to examine the impact of different freezing patterns on the resulting mechanical properties and morphology of the monoliths.

Prior to use, the polytetrafluoroethylene (PTFE) molds were cleaned and dried. 140 µL of type I collagen microfibrillar derived from bovine tendon (Advanced BioMatrix) dispersion was pipetted into each well and the mold was then placed inside a temperature-controlled shelf freeze-dryer (Advantage EL-85, SP Scientific) for freeze-casting treatment.

As for the controlled freezing rate method, the temperature-controlled shelf freeze-dryer

(Advantage EL-85, SP Scientific) was first set to 5°C. The shelf temperature was then ramped down to -20°C at a known rate (0.2°C/min, 2°C/min, 5°C/min, 10°C/min). Upon freezing, a vacuum of <0.10 mbar was set and the shelf temperature was kept at -20°C. The samples were dried for 24h before removing them from the dryer. Upon the removal, the samples were crosslinked with 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide hydrochloride (EDC) (Sigma-Aldrich, UK) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich, UK) for 24h. After the crosslinking, all the samples were freeze-cast again as the first freezing cycle. Collagen scaffold were stored in the desiccator and kept at room temperature until needed.

As for the controlled nucleation temperature method, the temperature-controlled shelf freeze-dryer (Advantage EL-85, SP Scientific) was first cooling down to -5°C, -10°C, -20°C, -30°C, -50°C. The shelf temperature was then kept at this constant temperature. After the shelf temperature was stabilized, Collagen solutions within Teflon mold were placed on the shelf. Upon freezing, a vacuum of <0.10 mbar was set and the shelf temperature was kept as constant. The samples were dried for 24h before removing them from the dryer. Upon the removal, the samples were crosslinked with 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide hydrochloride (EDC) (Sigma-Aldrich, UK) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich, UK) for 24h. After the crosslinking, all the samples were freeze-cast again as the first freeze-casting cycle. Collagen scaffold were stored in desiccator and kept at room temperature until needed.

By use of the freeze-casting techniques, various collagen scaffold with different porosity were produced. Scaffolds were fabricated from a suspension of type I collagen microfibrillar derived from bovine tendon (Advanced BioMatrix) at a concentration from

0.5 wt.% to 1.25 wt.%. The suspension of type I collagen was prepared in 0.05 M acetic acid solution (Sigma-Aldrich), and the pH was adjusted to 2.0 with hydrochloric acid (VWR International). The collagen suspension was blended by an overhead homogenizer (T 10 BASIC S001) for 30 min at cold water bath and then, after mixing, centrifuged at 2500 rpm for 10 min to remove the air bubbles. The type I collagen suspension was poured into rectangular Teflon molds, 10×7×2 mm^3 in size. The samples were frozen in a freeze-dryer (VirTis Advantage Plus ES; SP Scientific; PA, USA) under constant temperature for 5 hours, and then dried at the same temperature under 10 mTorr for 24 hours.

Also, the impact of additives such as gelatin (bovine skin, Sigma-Aldrich) was investigated in the freeze-cast dispersions. A known mass of gelatin was added into the collagen dispersion prior to the freeze-casting and the mixture was sonicated for 5 minutes before use. 140 µL of dispersion was pipetted (per well) for freeze-casting in a PTFE mold.

2.6.2 Structural Characterization of Type I Collagen Scaffolds

The structural of collagen scaffolds were characterized by scanning electron microscopy (SEM) and mercury intrusion porosimetry **[32]**. SEM (Mira3 FESEM, Tescan) was performed on Au/Pd sputtered samples at 15 keV. The pore sizes and distribution of the monolith samples were characterized by Hg intrusion porosimetry (Autopore IV 9500; Micromeritics). Monoliths were placed in glass penetrometers of known weight and volume, which were then mounted horizontally into the instrument. Analyses were performed using an automated procedure in which penetrometers are first evacuated to 50 µm Hg and then filled with Hg at 0.2 psi. Pressure applied to the column of Hg was ramped stepwise to 48 psi to probe pores with diameters greater than 4 µm in diameter. Monoliths were also characterized with a dynamic mechanical analyzer (DMA Q800; TA Instruments)

to characterize the impact of collagen concentrations on scaffold mechanical properties. Collagen scaffolds were placed under the compressive loads after being soaked in PBS solution under room temperature overnight. Measurements were comprised of a single loading cycle, with an initial contact force of 0.05 N and a ramp rate of 0.005 N/min to 0.075 N. Elastic modulus for the monoliths was calculated as the slope of the stress-strain curve over the 1-2% strain regime **[33]**.

3. Results

3.1 Influence of Controlled Freezing Rate on Scaffold Morphology

Previous studies show that the morphology of freeze-cast collagen monoliths is strongly dependent on the selection of mold and freezing sequence applied. According to the previous study in our group, samples freeze-cast in PTFE molds generally exhibit higher pore volumes than their counterparts freeze-cast in brass molds.

The morphology of various collagen scaffolds, obtained by scanning electron microscopy, is shown in **Figure 3.1.1**

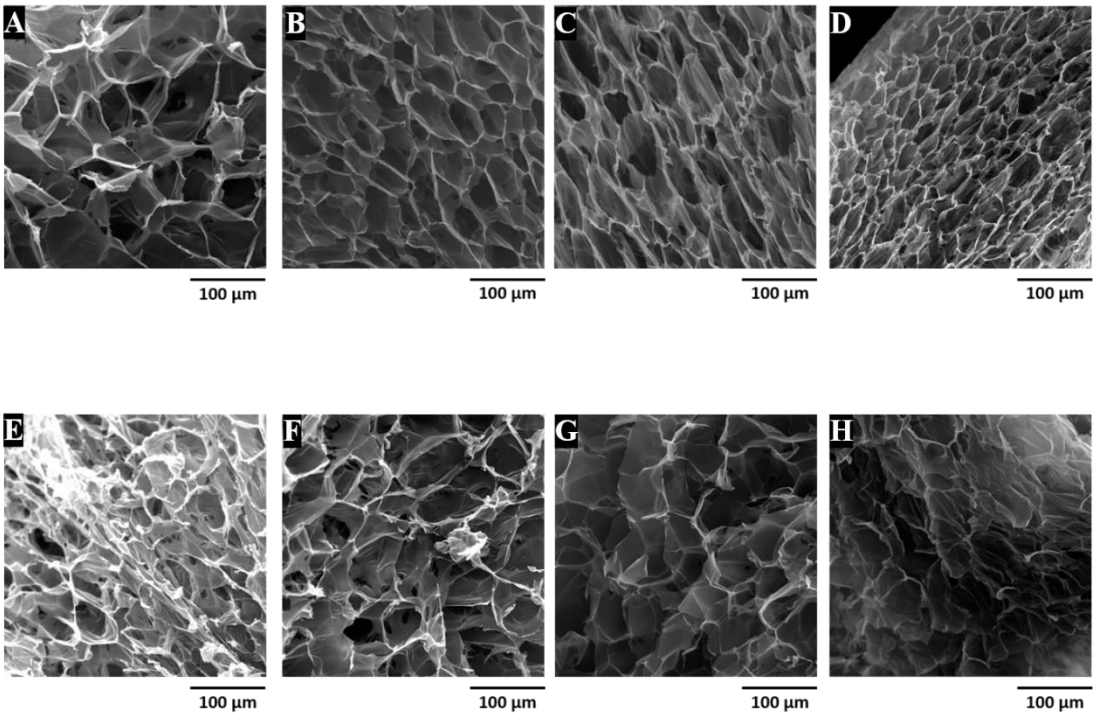

Figure 3.1.1: SE micrographs of collagen scaffolds with controlled freezing rate. A: 0.5 wt.%-0.2°C/min; B: 0.75 wt.%-0.2°C/min; C: 1.0 wt.%-0.2°C/min; D: 1.25 wt.%-0.2°C/min; E: 0.5 wt.%-5°C/min; F: 0.75 wt.%-5°C/min; G: 1.0 wt.%-5°C/min; H: 1.25 wt.%-5°C/min. Scale bar: 100 µm

Samples were freeze-cast in PTFE molds at slow (0.2°C/min) and high (5°C/min) ramp rates from 5°C to -20°C. Scanning electron (SE) micrographs of samples freeze-cast at low (0.2°C/min) and high ramp rates (5°C/min) are shown in **Figure 3.1.1** For all the ramp

rates observed, the use of PTFE molds led to the formation of rounded pores. Freeze-casting of the dispersions at higher collagen concentrations led to the formation of the structures with smaller pore sizes. Freeze-casting of the dispersions at higher freezing rate or lower freezing rate could not led to the formation of the structures with significant differences. Under these conditions, monoliths prepared in PTFE molds with the lowest collagen concentrations (0.5 wt.%) and the lowest freezing rate (0.2°C/min) offered the largest pore volume and pore diameter out of all samples studied.

Table 3.1.1: Elastic modulus (in compression) of freeze-cast samples prepared with different collagen concentrations and freezing rate

Samples	Average Elastic Modulus (Pa)
1%-0.2°C/min	2251
1%- 2°C/min	2455
1%-5°C/min	1779
1%-10°C/min	2339
1.25%-0.2°C/min	2433
1.25%-2°C/min	2987
1.25%-5°C/min	2619
1.25%-10°C/min	2733

The influence of Controlled Freezing Rate on the compressive modulus of collagen scaffolds was studied by dynamic mechanical analysis, as shown in **Table 3.1.1**, representative stress-strain profile is shown in **Figure 3.1.2**

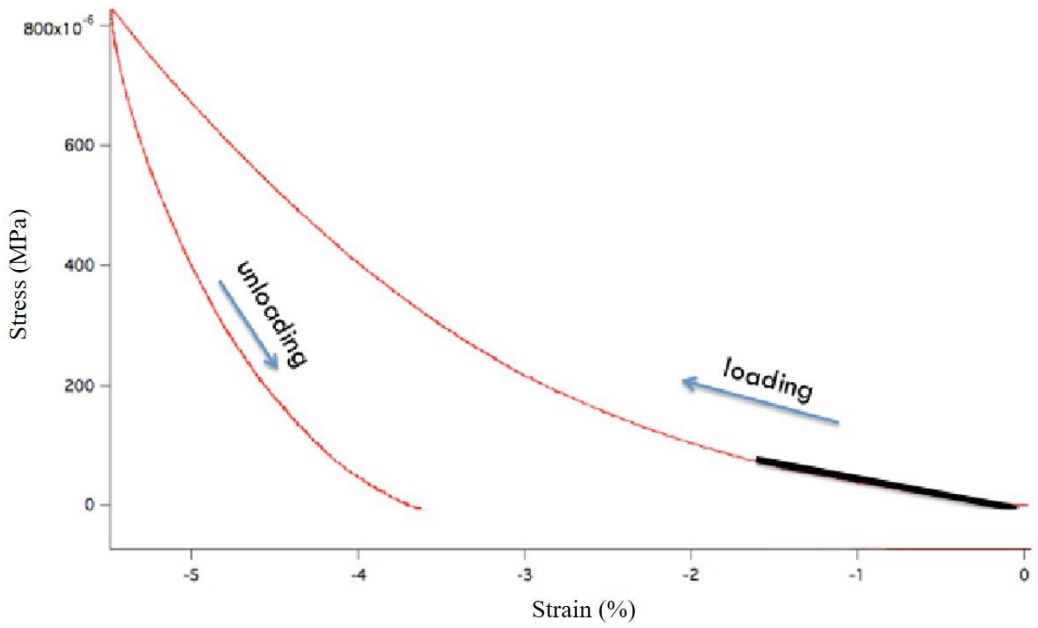

Figure 3.1.2: Representative stress-strain curve of sample in compression test. Elastic modulus taken as slope at low strain, indicated by black line [4]

Compared among all the samples, it is clear that controlling the freezing rate could not lead to significant differences in elastic moduli.

3.2 Influence of Controlled Nucleation Temperature on Scaffold Morphology

With the three types of scaffold mold and controlled nucleation temperature method, here, the images of the collagen scaffold made form the above-mentioned method are shown in **Figure 3.2.1**, from the left to the right, rectangular thin scaffold, circular thick scaffold, and rectangular thick scaffold.

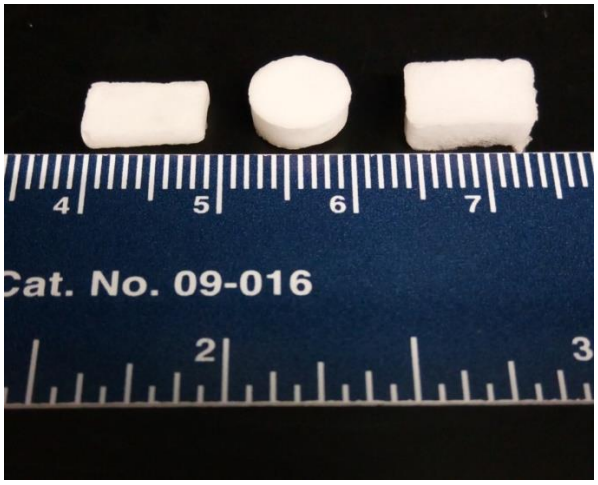

Figure 3.2.1: Representative samples of collagen scaffolds with controlled nucleation temperature method

Representative samples and SE micrographs of collagen scaffolds with controlled nucleation temperature at -5℃ are shown in **Figure 3.2.2**

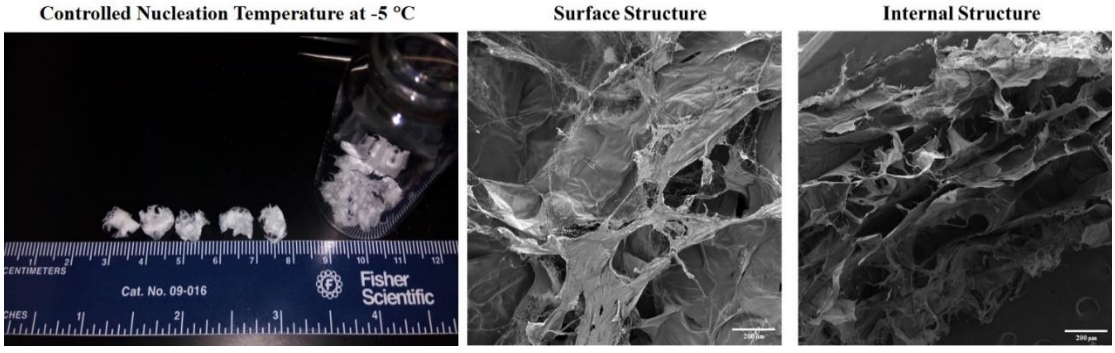

Figure 3.2.2: Representative samples and SE micrographs of collagen scaffolds with controlled nucleation temperature at -5℃

The ice crystals in both thick molds grow too large when the collagen scaffolds were fabricated with controlled nucleation temperature at -5℃, so the overall shape of the collagen scaffolds were hard to control. Most of the scaffolds seemed “exploded” from the inside structure.

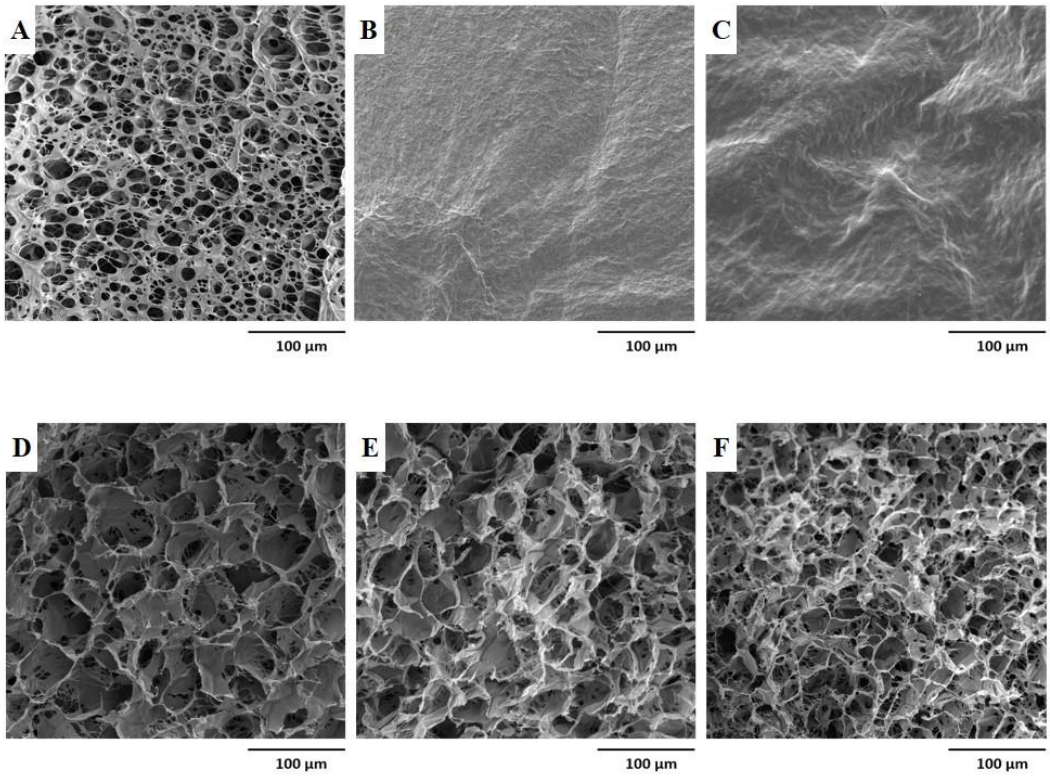

Figure 3.2.3: SE micrographs of collagen scaffolds with controlled nucleation temperature at -5℃. A: Surface-0.75 wt.%; B: Surface-1.0 wt.%; C: Surface-1.25 wt.%; D: Internal-0.75 wt.%; E: Internal-1.0 wt.%; F: Internal-1.25 wt.%; Scale bar: 100 µm

Top surface and internal structure scanning electron (SE) micrographs of samples freeze-cast with controlled nucleation temperature at -5℃ are shown in **Figure 3.2.3**. For all the samples observed, the thin scaffolds could still retain their intact structure within the mold, and interestingly, the pore size at top surface was drastically smaller than the internal pores and both decreased with the increased collagen concentrations.

The possible explanation for this phenomenon is due to the sharp temperature gradient, the number of ice nucleation sites at the air interface were much higher than the internal structure, so the pore size at the scaffold top surface (air interface) was much smaller than the pore size within the bulk structure.

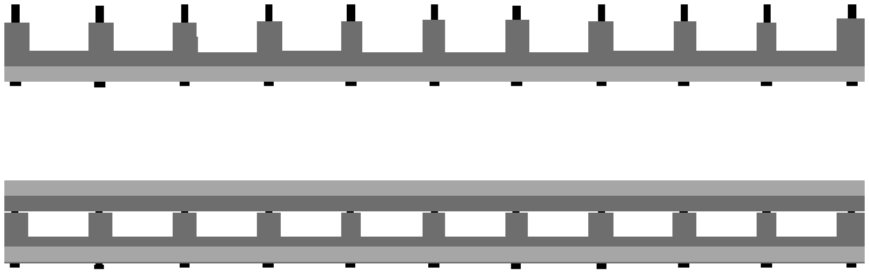

Figure 3.2.4: Lateral View of Rectangular/Circular Thick Mold

In order to increase the pore sizes at the air interface, another layer of Teflon plate was applied onto the top surface both rectangular and circular thick mold. Thus, this "Sandwich Configuration" may help to reduce the temperature gradient and increase the open pore sizes at the top surface of the scaffolds.

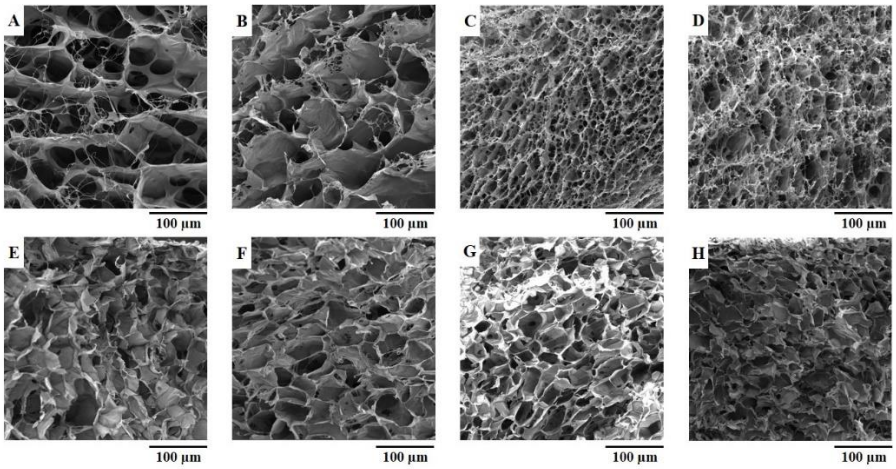

Figure 3.2.5: SE micrographs of collagen scaffolds with controlled nucleation temperature at -10°C. A: Surface-0.5 wt.%; B: Surface-0.75 wt.%; C: Surface-1.0 wt.%; D: Surface-1.25 wt.%; E: Internal-0.5 wt.%; F: Internal-0.75 wt.%; G: Internal-1.0 wt.%; H: Internal-1.25 wt.%; Scale bar: 100 µm

After the Teflon layer was applied onto the top of scaffold molds, here the SEM image of Collagen Scaffolds in the Rectangular Thick Mold at -10℃ with controlled nucleation temperature method are shown in **Figure 3.2.5**, the surface pore size increased significantly and there were much more open pores at the air interface, this phenomenon was attributed to the top Teflon layer that reduced the freezing temperature gradient, so "Sandwich Configuration" could get much more open and large pores at the top surface of collagen scaffolds.

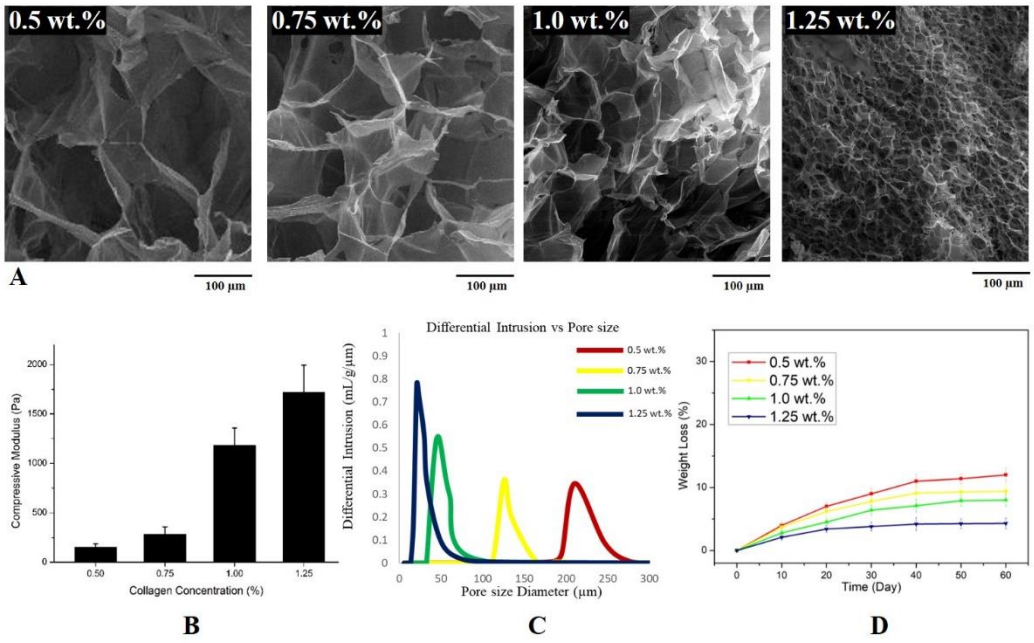

Figure 3.2.6: A: SE micrographs of collagen scaffold at -10℃; Scale bar: 100 μm

B: Compressive modulus of rectangular thin collagen scaffolds with different collagen concentration at different freezing temperature

C: Pore size distribution of the type I collagen scaffolds with four different concentrations. Red: 0.5 wt.%, Yellow: 0.75 wt.%, Green: 1.0 wt.%, Blue: 1.25 wt.%

D: Degradation test of the type I collagen scaffolds with four different concentrations. Red: 0.5 wt.%, Yellow: 0.75 wt.%, Green: 1.0 wt.%, Blue: 1.25 wt.%

Figure 3.2.6 shows the summary of collagen scaffolds fabricated in rectangular thin mold (without Teflon top layer) with controlled nucleation temperature at -10℃. The porous

structure of scaffolds was shown in SE micrographs, with collagen concentration increased, the average pore size decreased from 220 μm (0.5 wt.%) to 30 μm (1.25 wt.%), and the porosity increased accordingly, based on ImageJ analysis. Additionally, the compressive moduli of collagen scaffolds increased with increasing collagen concentration from 0.5 wt.%. to 1.25 wt.%. The highest compressive modulus measured was on the 1.25 wt.% collagen scaffold, which was measured as 1720 ± 300 Pa. This is a 11-fold increase compared to the modulus of the 0.5 wt.% collagen at same freezing condition. The pore structure of scaffolds was also investigated by Hg intrusion porosimetry. The pore size distribution of these freeze-casting structures is shown in **Figure 3.2.6 C**. It showed that the average pore size decreased significantly with the increased collagen concentration from 214 μm (0.5 wt.%) to 35 μm (1.25 wt.%). The largest pore size was found on the 0.5% collagen scaffold with an average of 214 μm. These 0.5% collagen scaffolds had a significantly larger pore diameter compared to the other three scaffold groups. The pore size distribution also showed clearly that higher collagen concentration will narrow down the pore size distribution which means the pore size becomes more uniform within these scaffolds. The samples were also characterized for degradation test, from the weight loss versus degradation time graph as shown in **Figure 3.2.6 D**, it is clear that, although all the samples showed good stability over sixty days, the highest collagen concentration led to the lowest weight loss.

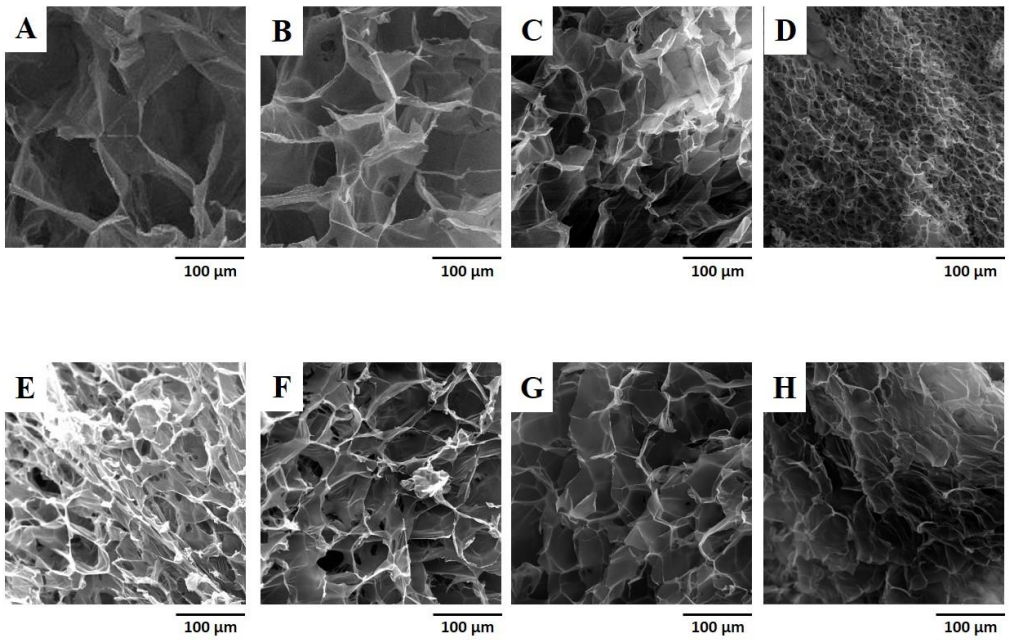

Figure 3.2.7: SE micrographs of collagen scaffolds with controlled nucleation temperature (CNT) and controlled freezing rate (CFR) at -10°C. A: CNT-0.5 wt.%; B: CNT-0.75 wt.%; C: CNT-1.0 wt.%; D: CNT-1.25 wt.%; E: CFR-0.5 wt.%; F: CFR-0.75 wt.%; G: CFR-1.0 wt.%; H: CFR-1.25 wt.%; Scale bar: 100 µm

The difference of collagen scaffolds made with controlled nucleation temperature (CNT) and controlled freezing rate (CFR) are summarized in Figure 3.2.7, both at -10°C. It is clear that, even at the same collagen concentration and final cooling temperature, the CNT method led to more pronounced pore sizes variations compared to the CFR method.

As the controlled nucleation temperature (CNT) method shows better performance over the controlled freezing rate (CFR) method, different mold design was used to explored the possible structure result from this method, the **Figure 3.2.8** shows the summary of collagen scaffolds made from rectangular thick mold and circular thick mold at -10°C.

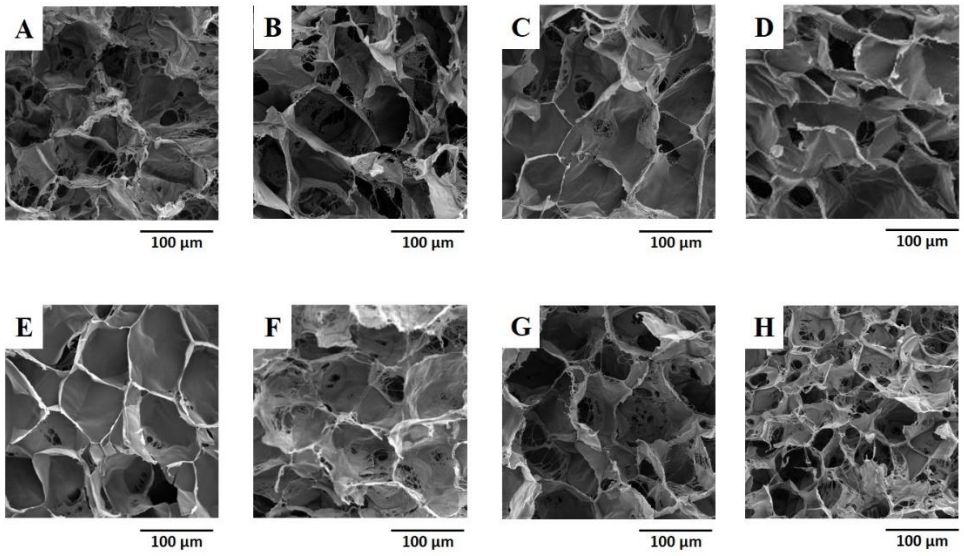

Figure 3.2.8: SE micrographs of collagen scaffolds with controlled nucleation temperature (CNT) at -10℃. A-D were made with rectangular thick mold. E-H were made with circular thick mold A: 0.5 wt.%; B: 0.75 wt.%; C: 1.0 wt.%; D: 1.25 wt.%; E: 0.5 wt.%; F: 0.75 wt.%; G: 1.0 wt.%; H: 1.25 wt.%; Scale bar: 100 µm

Interestingly, when the controlled nucleation temperature (CNT) method was used with both rectangular thick and circular thick mold as shown in **Figure 3.2.8**, the pore size difference along with different collagen concentration is not as clear and obvious as the samples made from the rectangular thin mold. Neither of the thick molds showed a clear pore size distribution.

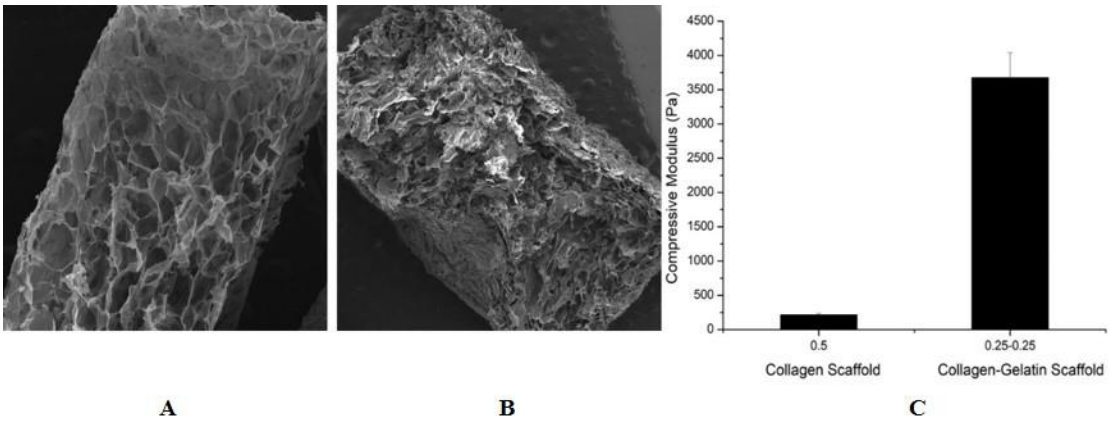

Figure 3.2.9: A: SE micrographs of 0.5 wt.% pure collagen scaffolds with controlled nucleation temperature (CNT) and at -10℃. B: SE micrographs of 0.25 wt. % collagen-0.25 wt. % gelatin scaffolds with controlled nucleation temperature (CNT) at -10℃. C: Compressive moduli of pure collagen scaffolds and collagen-gelatin scaffolds

Another natural polymeric protein, gelatin was added into the collagen solution to investigate the relationship of gelatin addition with the collagen scaffold morphology **[34]**. 0.5 wt.% pure collagen scaffolds and 0.25 wt. % collagen-0.25 wt. % gelatin scaffolds were prepared. The SEM images in **Figure 3.2.9** showed that after the gelatin addition, the scaffolds pore size decreased significantly and could not lead to a well-established structure. However, even at the same solid content condition, the gelatin addition into the collagen-gelatin scaffold could highly increase the scaffold mechanical moduli compared to the same pure collagen scaffolds.

3.3 Use of Collagen Scaffolds for Mechanobiology Study

To evaluate the use of collagen scaffolds for cell culture experiments, 3T3-L1 mouse fibroblast cells were seeded into various matrices. 3T3-L1 cells were chosen because of their highly activity during migration and matrix assembly stage. Briefly, the scaffolds were cut into 1 mm width and individually placed into the wells of a Labtek^{TM} (Thermo Fisher). With the transverse section of the scaffolds faced upside, cells were seeded onto the top of the scaffolds in 1% FBS αMEM. The scaffolds were incubated for an hour for the cells adhesion, after that the fresh media was added into the wells to promote the cell growth. The scaffolds were then placed into the incubator for 24 hours **[35]**.

To evaluate the cell adhesion on scaffolds with different micro-structure, the scaffold-bound cell populations were characterized after 24 hours of cell culture. The fluorescent assay of fixed scaffolds showed that most of the cells established different extent of

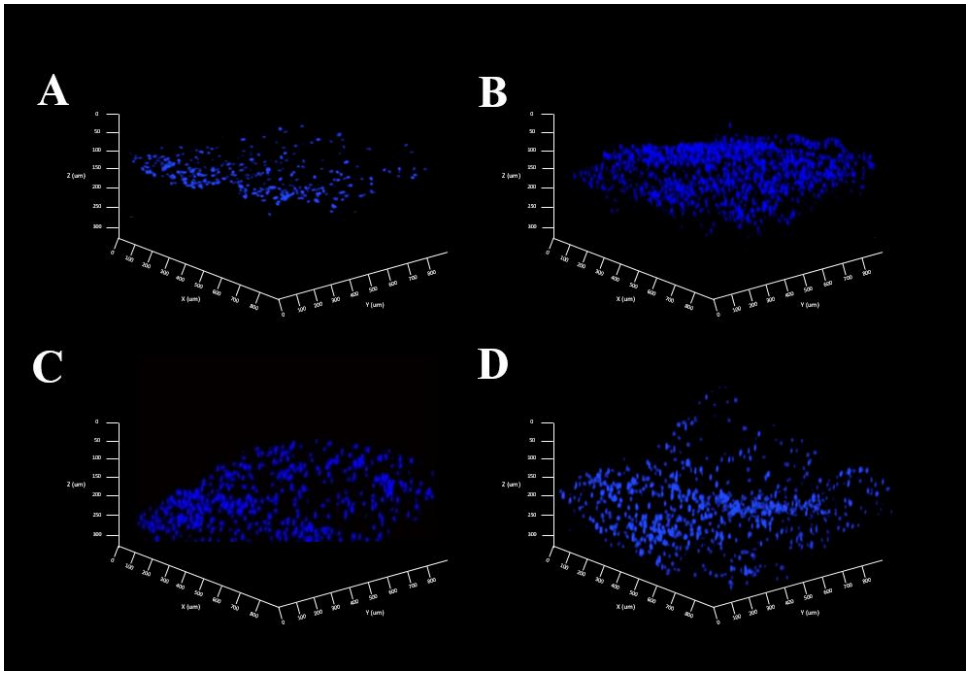

Fig. 3.3.1: Fluorescence micrograph of collagen scaffolds, with cell nuclei are stained with DAPI (blue) after 24 hours of 3T3-L1 culture. Z-stacks were taken from the top of the scaffolds. (A) 0.5 wt.% collagen scaffold (B) 0.75 wt.% collagen scaffold (C) 1 wt.% collagen scaffold (D) 1.25 wt.% collagen scaffold

adhesion on collagen scaffolds with different pore sizes and stiffness after short-term (24 hours) culture. The cell invasion results indicated that cells established better adhesion and migration ability in the scaffolds with higher collagen concentration, which also had smaller pore size. In 0.5 wt.% (**Figure 3.3.1 A**) and 0.75 wt.% (**Figure 3.3.1 B**) collagen scaffolds, most of the cells were adhered on the top of the scaffolds, while 0.75 wt.% collagen scaffolds showed higher cells quantity. In 1% (**Figure 3.3.1 C**) and 1.25 wt.% (**Figure 3.3.1 D**) collagen scaffolds, cells invaded through the scaffolds. Furthermore, we collected more data by investigating the cell adhesion on the top, middle and bottom of the scaffolds. Interestingly, our results indicated that, although cells invaded through both collagen scaffolds (1 wt.% and 1.25 wt.%), fewer cells adhered on the middle of the 1% collagen scaffolds, from which we inferred that the pore sizes in the middle of the scaffolds may be larger than normal. These confocal images indicated that 3T3-L1s successfully invaded the 1.25 wt.% collagen scaffold and adhered to the 3D collagen structure, which we decided to use the 1.25 wt.% collagen scaffolds for the further experiments.

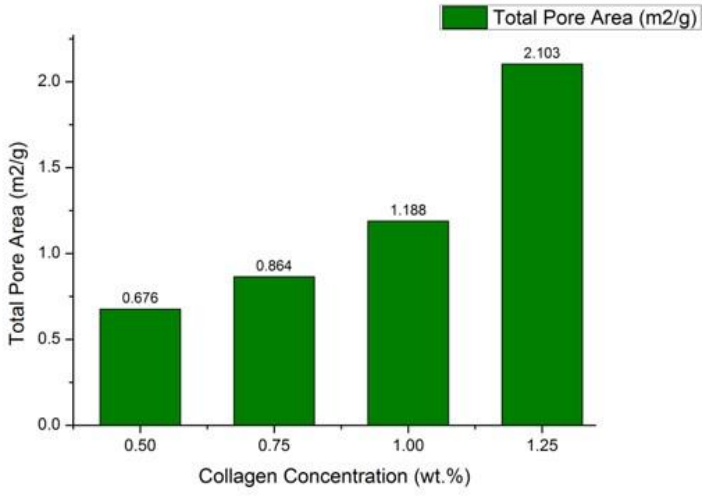

Figure 3.3.2: Total pore area of the type I collagen scaffolds with four different concentrations. Total pore area expressed in (m^2/g).

A possible explanation for the collagen scaffolds with 1.25 wt.% collagen concentration shows the best overall cell invasion results is that the 1.25 wt.% collagen scaffolds has the highest total pore area compared to the other 3 collagen groups, which is obtained from the Hg porosimetry (**Figure 3.3.2**).

3.4 Cell Viability and Matrix Deposition Capability

The fluorescent viability assay of unfixed collagen scaffolds confirmed that most of the cells were viable in the 3D collagen structure after short-term (24 hours) and long-term (6 days) culture (**Figure 3.4.1**) **[36]**. To investigate whether the 3T3-L1s functioned regularly, for instance, being able to assemble extracellular matrix (ECM), fluorescent labeled Fn was utilized to visualize the cell-deposited fibronectin (Fn) fibers. As shown in the image, Fn not only decorated the surfaces of the scaffold, but was also assembled into the thin fibrils (with higher brightness) around the periphery of cells (**Figure 3.4.1 C**), indicating the ability of the cells to polymerize Fn molecules into Fn fibers. These findings suggested that the 1.25% collagen scaffolds provide a satisfying 3D environment for fibroblasts to attach, grow, and functioning normally, which fulfilled the essential criteria for an in-vivo mimicking 3D cell-culture platform based on type I collagen.

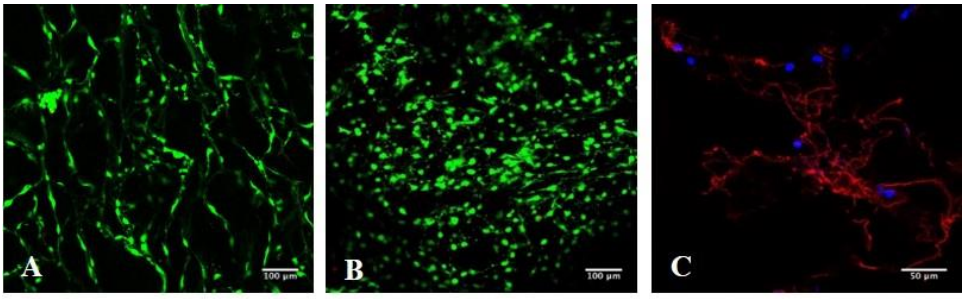

Figure 3.4.1: (A) Fluorescence micrograph of collagen scaffold after 24h of 3T3-L1 culture. (B) Fluorescence micrograph of collagen scaffold after 7 days of 3T3-L1 culture, showing very high cell viability. Live cells are stained with calcein (green), and dead cells are stained with propidium iodide (red). (C) Fluorescence micrograph of collagen scaffold after seeding 3T3-L1 cells and incubated with media containing labeled Fn. Cell-deposited fibronectin fibers (red) after 24 hours of 3T3-L1 culture. Cell nuclei are stained with DAPI (blue).

4. Summary/Conclusions

3D collagen scaffolds with tunable porosity, mechanical properties were prepared via the freeze-casting of type I collagen dispersions. The impacts of freezing rate, mold shape and material composition, and dispersion concentration on the structure and mechanical properties were methodically explored. Coarse control of the pore size distribution was achieved by changing the thickness of scaffold molds and by varying the rate at which the dispersion was frozen. The "controlled nucleation temperature" method was the most effective way to fabricate collagen scaffolds with varied average pore size. Different nucleation temperatures led to different scaffolds morphologies and stiffness. Addition of gelatin proteins significantly increased the compressive modulus and reduced the scaffold pore size.

For cell studies, 1.25 wt.% type I collagen dispersions freeze-cast in PTFE molds offered the most appropriate pore size and morphology for cell invasion and proliferation. Incorporating EDC/NHS into the dispersion after freeze-casting improved the mechanical robustness of the scaffolds and enabled their use for longer-term cell culture. In addition, the crosslinked samples were successfully used to control the cell adhesion. The observed stability of the crosslinked collagen scaffold renders them good candidate materials for cell culture where long term scaffolding is needed, fine-tuning of their stability may be achieved by varying crosslinking conditions as previously discussed. Furthermore, the elastic modulus of the scaffolds can be significantly enhanced through the addition of chemical crosslinkers, consequently, the tunable morphology/mechanical properties of freeze-cast type I collagen scaffolds make them excellent candidate materials for tissue engineering and mechanobiology studies.

5. Opportunities for Future Study

The tunable porosity obtained by freeze-casting of type I collagen scaffolds makes them good candidates for cell culture and biomechanics studies. Preparing monoliths that have appropriate pore structure, pore connectivity and mechanical properties may present unique challenges, since changing the composition of the collagen dispersion prior to freeze-casting influences the morphology and stiffness of the prepared scaffolds. Incorporating water-dispersible components into the scaffold prior to freeze-casting may allow for structural reinforcement while mitigating the extent to which the size of the resulting macropore is altered.

Our research lays the groundwork for future investigation of the different methods to fabricate tunable collagen scaffolds and control the synergistic effects of collagen with other proteins present in the cellular/tissue micro-environment, which are crucial to govern cell-matrix interactions in mechanobiology studies. Regarding the combination of collagen with fibronectin, preliminary experiments showed that synergetic effect of collagen and fibronectin could promote the cell proliferation within the matrices.

Further work would be focused on (i) the development of short-term degradable collagen scaffolds for wound healing applications, (ii) the combination of type-I collagen solution together with fibronectin to fabricate collagen-fibronectin scaffolds that better mimic the natural extracellular matrix. As for the crosslinking process, UV-light and dehydrothermal treatments could be used to evaluate the crosslinking effect on collagen scaffold samples.

6. References

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